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AWARD NUMBER DAMD17-96-1-6088

TITLE: Identification of Two Candidate Tumor Suppressor Genes on
Chromosome 17p13.3: Assessment of Their Roles in Breast and
Ovarian Carcinogenesis

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REPORT DATE: July 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
               U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
                         distribution unlimited

19971218 014

DTIC QUALITY INSPECTED 4

REPORT DOCUMENTATION PAGE

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE July 1997	3. REPORT TYPE AND DATES COVERED Annual (1 Jul 96 - 30 Jun 97)

4. TITLE AND SUBTITLE
Identification of Two Candidate Tumor Suppressor Genes on Chromosome 17p13.3: Assessment of Their Roles in Breast and Ovarian Carcinogenesis

5. FUNDING NUMBERS
DAMD17-96-1-6088

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Breast cancer is one of the most common malignancies affecting women in the US. Despite the magnitude of this disease little is known about the molecular events that occur during its development. We have identified a region of less than 30 kbp, on chromosome 17p13.3 by allelic loss mapping, which is altered in >50% of the breast tumors analyzed. Using positional cloning techniques we have identified a gene, OVCA2, within this region, which we believe may be a tumor suppressor gene. We have found that OVCA2 (1) is a novel gene residing in a chromosomal region which is frequently lost in breast, brain, colon, and ovarian tumors, (2) is highly conserved, (3) is expressed in all tissues, but appears to be lower in breast tumors, relative to normal mammary epithelial cells, and 4) interacts with several key proteins, some which are involved in control of cell growth and morphology. The recent detection of an inactivating mutation in a breast carcinoma suggests that OVCA2 may play a role in the development of this disease. Identifying the role of OVCA2 in the pathogenesis of breast cancer might ultimately help us to better understand the disease and to plan more effective treatment strategies.

14. SUBJECT TERMS
growth suppressor, allelic loss, mutation analysis, alternative splicing, positional cloning, protein-interactions , breast cancer

15. NUMBER OF PAGES
20

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT unclassified	20. LIMITATION OF ABSTRACT unlimited

FOREWORD

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_____   __7/30/97____
Principal Investigator's Signature   Date

A. K. Godwin, Ph.D.

Table of Contents

Identification of Candidate Suppressor Genes on Chromosome 17p13.3: Assessment of Their Roles in Breast and Ovarian Carcinogenesis"

INTRODUCTION:

Breast cancer is one of the most common malignancies affecting women and is diagnosed in approximately 180,000 women each year in the United States (1). Despite the magnitude of this disease little is known about the molecular events that occur during its development. Alteration of chromosome 17 has been consistently demonstrated in breast tumors (2-5). Chromosome 17 has a number of potential cancer causing genes, including *TP53* at 17p13.1, the *BRCA1* gene at 17q21, *prohibitin* and *NM23* at 17q23-24, *NF1* at 17q11 and the proto-oncogene *ERBB2* at 17q21. Mutations in *BRCA1* have been detected in familial forms of breast and ovarian cancer, but are uncommon in sporadic forms of the diseases (6-10). *TP53* has also been implicated in the pathogenesis of breast cancer (11). Mapping of LOH on chromosome 17p however has identified an additional region, telomeric to *TP53* at 17p13.3, that is thought to harbor a tumor suppressor gene(s) involved in the pathogenesis of breast cancer (2). LOH at 17p13.3 has also been reported in ovarian tumors (12), primitive neuroectodermal tumors (13), carcinoma of the cervix uteri (14), medulloblastoma, osteosarcoma (15), and astrocytoma (16), suggesting that genes on 17p13.3 may play a role in the development of a wide variety of cancers.

BODY:

Identification of genes in a minimum region of allelic loss on 17p13.3. We have identified a minimum region of allelic loss on chromosome 17p13.3, between polymorphic markers D17S5 and D17S28, in genomic DNA from breast and ovarian tumors (Figure 1 and data not shown). Cosmid clones overlapping the minimum region of allelic loss were identified using the polymorphic markers as probes. Two of these cosmid clones suppressed clonal outgrowth when expressed in A2780 ovarian cancer cells (Figure 2), confirming the potential presence of a tumor suppressor sequence(s) in this minimum region of allelic loss. We have recently reported the identification of two novel genes, which we refer to as *OVCA1* and *OVCA2* (originally designated *TP60*), that map to our minimum region of allelic loss on 17p13.3 (17). The two genes are ubiquitously expressed and encode proteins of 443 and 227 amino acids, with no known functional motifs. Comparison of genomic and cDNA sequences showed that the genes overlap one another in the minimum region of allelic loss, and have one exon in common (Figure 3). However, sequence and Western blotting indicate that translation of *OVCA1* does not proceed into the shared exon, predicting the translation of completely distinct OVCA1 and OVCA2 proteins.

Tissue expression and evolutionary conservation of *OVCA2*. Multiple tissue Northern blots probed with a cDNA fragment containing the shared exon of *OVCA1* and *OVCA2* revealed that both transcripts are ubiquitously expressed in all normal human tissues tested. The relative amounts and the ratio of the expressed transcripts is tissue specific (Figure 4).

When zoo blots comprised of EcoRI fragments of genomic DNA from various species were probed with the unique exon 1 of *OVCA2*, hybridizing bands were visualized in every species tested (Figure 5). This indicates a high degree of evolutionary conservation of the gene. In addition, amino acid comparisons with GenBank/EMBL and Swissprot sequences revealed 1 nematode and 4 yeast proteins with significant amino acid similarity (up to 60%) to OVCA2 (Figure 6). These sequences were identified as putative DHFRs, but share more domains with OVCA2 than with mammalian DHFRs. A database search of expressed sequence tags (ESTs) identified partial expressed sequences for putative rat and mouse OVCA2 homologs (86, 87% similarity) (Figure 6), as well as two

plant ESTs (rice and arabidopsis, 53% similar). A multiple sequence alignment of OVCA2 with all similar sequences revealed at least 5 conserved domains, which have no known function, but which may be important new functional domains based on their evolutionary conservation. Thus, *OVCA2* is ubiquitously expressed and highly evolutionarily conserved, suggesting that it may be important for normal cellular function.

Alterations of *OVCA2* in breast cancer. Thirty seven primary breast tumors have been analyzed for LOH on 17p. 16 of these (43%) show LOH for polymorphic markers intragenic to *OVCA1* or *OVCA2*. 50% of these LOH positive tumors do not show LOH at the *TP53* locus (Table 1), indicating that a gene distal to *TP53* on 17p13.3 probably plays a role in breast cancer development. The high percentage of LOH in breast and ovarian tumors from patients unselected for family history implies that the genes on 17p13.3 may be involved in sporadic breast/ovarian cancer. This is in contrast to the breast/ovarian cancer susceptibility genes, *BRCA1* and *BRCA2*, which are primarily involved with inherited breast cancer, but probably do not play a role in the 85-90% of breast cancer cases which are sporadic (9, 18, 19). Functionally, 4 out of 6 breast tumors showed reduced mRNA levels relative to normal breast tissue, in slot blots of total RNA hybridized with a cDNA probe for the *OVCA2/OVCA1* shared exon (not shown). In addition, *OVCA1* and *OVCA2* mRNA levels were significantly reduced or undetectable in a high percentage of ovarian tumors and tumor cell lines (17). At least one mutation in exon 2 of *OVCA2* in an invasive ductal carcinoma of the breast has been detected using RT-PCR/SSCP analysis. Sequencing of this region showed that a portion of exon 11 of *OVCA1* had been inserted into exon 2 of *OVCA2* (Figure 7). This result was confirmed by Southern blot analysis, where an extra *EcoRI* restriction fragment could be detected with probes for *OVCA2* exon 2 and the full-length *OVCA1* (Figure 8). In addition, *OVCA2*'s promoter elements reside in a frequently methylated region of chromosome 17, and appear to be partially methylated (not shown), offering a second explanation for reduced mRNA levels in breast and ovarian tumors. Thus, a high frequency of LOH on 17p13.3, a reduction in mRNA levels, and an insertional mutation in *OVCA2* have all been shown in breast tumors, strengthening the relationship between loss of OVCA2 function and breast cancer development.

OVCA2 motif identification. Using the Wisconsin GCG package, we have identified 4 phosphorylation sites in OVCA2 (one in exon 1 and three in exon 2), two of which are conserved in the mouse and rat (Figure 6). In addition, a *MYB* DNA-binding motif was identified, which is identical to the native *MYB* motif, except for a conservative amino acid change from tryptophan to phenylalanine in position 1 (Figure 6). Interestingly, a casein kinase-2 phosphorylation site lies within this putative *MYB* DNA-binding domain, which could regulate its function. OVCA2 also contains a pseudo-leucine zipper motif and a proline rich hydrophobic region, both of which suggest that OVCA2 may interact with other proteins (see below).

Antibodies to OVCA2. We have attempted to generate polycolonal antibodies to OVCA2 in the following ways. Rabbits have been injected with several different 15 amino acid peptide portions of OVCA2. These antibodies have been purified on immunoaffinity columns made by linking the immunogenic peptide to agarose resin. In addition, we have expressed OVCA2-GST fusion proteins in bacteria, which we have also used to immunize rabbits. All these antibodies recognize OVCA2 as a 24-29 kDa protein when expressed in Cos-1 cells, and we are now in the process of characterizing the specificity of several of these OVCA2 antibodies. At least one of the antibodies (OV2-3) directed against a C-terminal OVCA2 peptide appears to specifically recognize bacterial and Cos-1 cell expressed protein, as well as endogenous OVCA2 in most of the normal tissue extracts tested (Figure 9 and data not included). In preliminary studies, OV2-3 also appear to weakly immunoprecipitate a slightly larger form of OVCA2 from nuclear protein extracts.

Intracellular localization of OVCA2. OVCA2 was transiently expressed in MCF7, MDA-MB468, PANC-1, SKOV3, A2780, and Cos-1 cells as a hemagglutinin (HA) fusion protein. The cells were grown on cover slips, and immunofluorescence (IF) was performed using a monoclonal antibody to the HA-tag. OVCA2 exhibited both a cytosolic

and nuclear localization as determined by confocal fluorescent microscopy, indicating that OVCA2 may reside in multiple compartments within the cell. This is consistent with results from a yeast two-hybrid screen, which indicate interactions with both cytosolic and nuclear proteins (see below). Intracellular distribution of OVCA2 is currently being explored further in the context of its interactions with other proteins and co-localization using antibodies to OVCA2 interactors.

Interactions of OVCA2 with other proteins. A yeast two hybrid/interaction trap approach (20) was employed to ascertain what proteins, if any, interact with OVCA2. Briefly, a fusion protein comprised of the entire coding region of *OVCA2* and the DNA binding domain (DBD) of LexA was expressed in yeast. The yeast contain *lacZ* (β-galactosides gene) and *LEU2* (required for leucine biosynthesis) reporter genes, both of which contain lexA operator sites. Stimulation of these genes through protein binding to lexA operators results in blue colony growth on X-gal-containing media and growth on leucine minus plates, respectively. The OVCA2/DBD bait protein cannot bind to the lexA operator unless it is in close proximity to a protein containing the lexA transcriptional activation domain (AD). The OVCA2/DBD containing yeast were transformed with an AD-HeLa cDNA fusion library. cDNA clones encoding proteins that specifically interacts with OVCA2 were identified as a result of transcriptional activation of the *LacZ* and *LEU2* genes. Specific interactors were scored by their ability to grow on media containing galactose lacking leucine and to modify the substrate X-gal (i.e., expression β-galactosidase) as compared to uninduced conditions (i.e., glucose containing media). We initially screened 244 doubly transformed yeast clones (Table 2). Of these, 100 showed stimulation of both the *LacZ* and *LEU2* genes (i.e., double positives) to varying degrees, with 54 clones being scored as strongly positive for both phenotypes. A second screen was employed to verify the clones that interact most strongly with the OVCA2 baits. Of these, two thirds have been sequenced yielding 22 unique known genes which are strong OVCA2 interactors (Table 3). Mutations are being introduced into OVCA2 at domains of interest and the interactions of each protein evaluated. We will extend our studies to determine the specificity of these interactions in mammalian cells by immunoprecipitations and co-localization approaches.

Tumor suppressor activity. While a causal relationship of altered OVCA2 expression and cancer pathogenesis has not been definitively established, it is clear from our preliminary studies that introduction of cosmids containing the entire *OVCA2* gene in tumor cell lines results in reduced cell growth and/or death. Furthermore, OVCA2 is expressed at higher levels in normal mammary epithelial and ovarian surface epithelial cells than many tumors and tumor cell lines. Since it is suspected that this underrepresentation of OVCA2 may be part of a pathway involved in tumor development, further insights can be gained by expressing the cDNA in multiple tumor cell lines. We have recently initiated studies to examine the effect of expression of exogenous OVCA2 on tumor cell growth. The purpose of these studies are to further investigate the growth suppression properties of OVCA2 and to help evaluate the function of OVCA2. *OVCA2* expression vectors are currently being stably transfected into multiple SV40-immortalized, non-tumorigenic and malignantly transformed cell lines and assessed for suppression of growth by direct cell counting and subsequently in soft agar assays. In order to make correlations between growth suppression and the levels of OVCA2 tumor cell lines, we have introduced a green fluorescent protein (GFP)/OVCA2 fusion construct into cell lines by transfection. This way the levels of OVCA2 expression can be observed in the living cells using a confocal microscope. Studies will be performed in parallel with cells transfected with C- and N-terminal HA tagged OVCA2, followed by immunofluorescence using an anti-HA antibody to ensure consistent localization and growth suppression. In order to begin to evaluate the nature of the growth suppression, deletion constructs will be used to determine which regions of OVCA2 are important for growth suppression. In addition we will perform experiments to determine if growth suppression is related to a state of replicative senescence. These experiments are of interest because the outcome will help address at

least three important questions: 1) is OVCA2 a classic tumor suppressor gene, 2) are changes in the level of expression of OVCA2 alone sufficient to markedly alter the transformed phenotype of both breast and ovarian cell lines, and 3) at what levels and how soon after introduction of OVCA2 does suppression of cell growth and reversion of the malignant phenotype occur.

Candidate disease-causing 17p13.3 genes. We have also continued our efforts to discover additional interesting genes in the area surrounding *OVCA1* and *OVCA2,* which may have relevence to cancer and human genetic disorders. It has been shown that this 17p13.3 region is highly conserved between human and mouse (21). It is also interesting that the *OVCA1/OVCA2* genes map to a region of the genome important in two well-characterized disorders: isolated lissencephaly sequence (ILS) and Miller-Dieker syndrome (MDS). Contiguous gene syndromes such as these are the consequence of *de novo* deletions of characteristic chromosomal regions, resulting in hemizygosity and haploinsufficiency of genes contained within the deleted regions. Additional gene(s) distal to *LIS1* on 17p13.3 may be responsible for the facial dysmorphology and other abnormalities seen in MDS but not in isolated lissencephaly sequence (ILS) patients. We have cloned more than 100 kbp centromeric to *OVCA1* and *OVCA2,* and are in the process of sequencing the entire genomic region. We have already identified one known and three previously unidentified genes. We hope in the near future (but not within the scope of this grant) to determine if *OVCA2* or any of the other recently identified 17p13.3 genes are viable candidates for the MDS gene. We will, however, be evaluating these new genes for deleterious mutations in cancers showing frequent genetic alterations (e.g., breast, brain, colon, and ovarian tumors). In this aspect we have perfected a novel mutation detection system referred to as MDA (mismatch detection analysis) that was developed in collaboration with Dr. Anthony Yeung at our Institute and a local biotechnology company. This highly sensitive method allows for detection of as little as a single base alteration in DNA fragments of greater than 1.5 kbp (Figure 11).

In Summary, *OVCA2* (1) is a novel gene residing in a chromosomal region which is frequently lost in breast and ovarian cancer, and in several other neoplasias, (2) is ubiquitously expressed and highly evolutionarily conserved, (3) is expressed at lower levels in human breast and ovarian cancer, and 4) interacts with several key proteins, some which are involved in control of cell growth and morphology. Thus, it may be very important in the pathogenesis of breast and ovarian cancer. Based on these observations, it is critical that we continue to characterize the normal function of OVCA2 and determine the biological consequences of OVCA2 inactivation with respect to breast cancer development. Specifically, it is essential that we investigate OVCA2's tumor suppressive activity, its tissue levels and intracellular distribution during cancer progression, and its biochemical mechanism of action. In this aspect we have made significant strides towards deriving antibodies that recognize OVCA2, determining its subcellular location, and identifying relevant proteins that interact with OVCA2.

ILLUSTRATIONS, FIGURES & tables
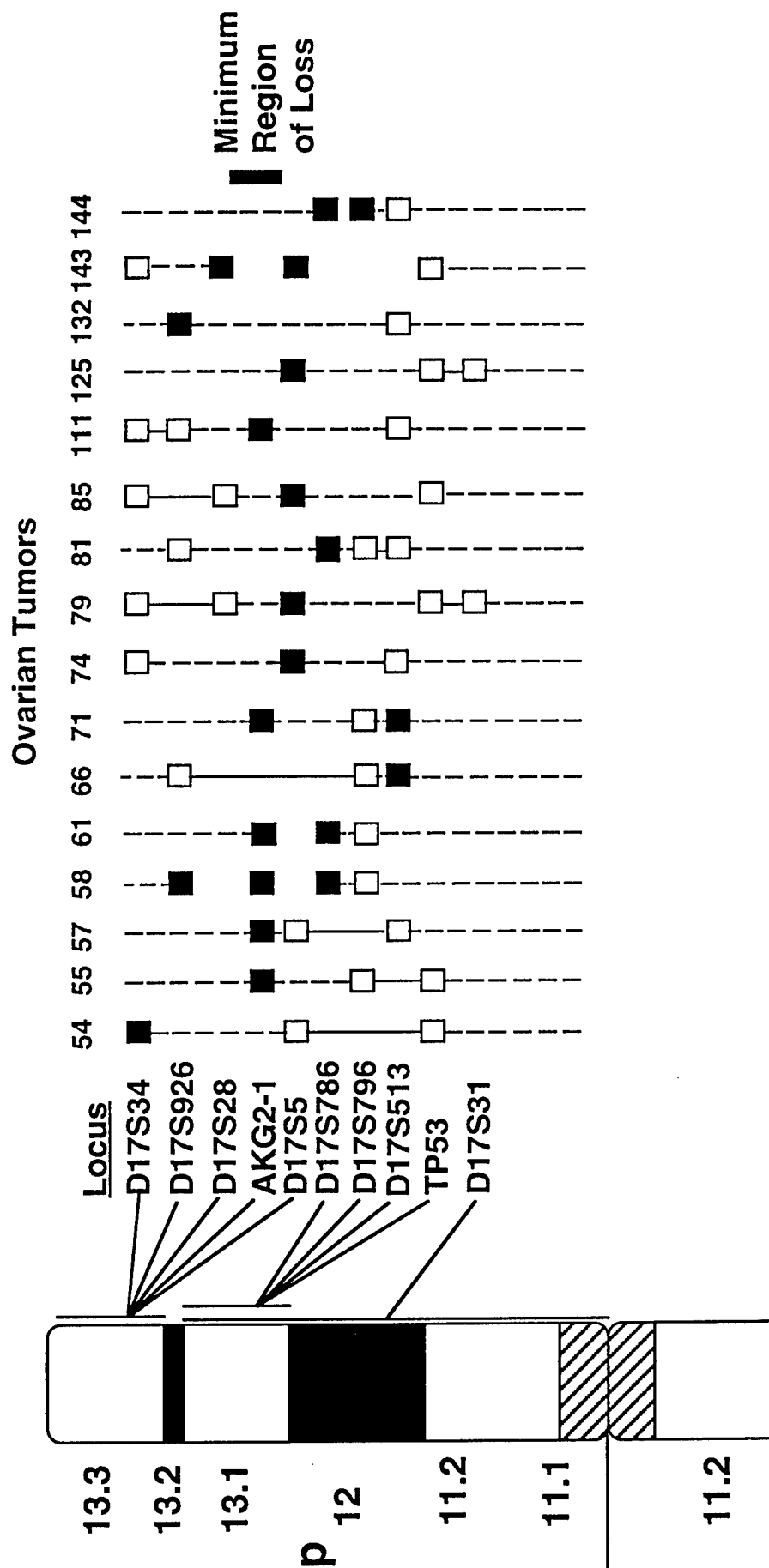
Figure 1. Allelic loss patterns of ovarian tumors for the short arm of chromosome 17. DNA samples from normal blood and ovarian tumor tissue were typed with STRPs from 17p. For each tumor, all informative loci are shown. Black squares represent constitutional heterozygosity with LOH; open squares, constitutional heterozygosity with no LOH; blank spaces, uninformative. With the assumption that alleles in all regions between loci showing allelic loss are lost, solid lines indicate retained regions of chromosome 17p and open areas represent regions of allelic loss. Dashed lines represent regions that are uncertain in tumors with loss of heterozygosity for some loci.

Minimal Region of Growth Suppression

Experiments	1	2	3
Placental DNA	82	91	98
pKOneo alone	87	79	102
Chrom 17p13.3 clones			
c1-2	79		
c2-1	35		
c3-1	89	81	75
c7-2	32	39	21
c15-1	88		
19-1	91		
Chrom 9q clones			
c62E9	75		

B=*BssH* II
E=*Eco* RI
N=*Not* I
S=*Sfi* I
*=Site in vector
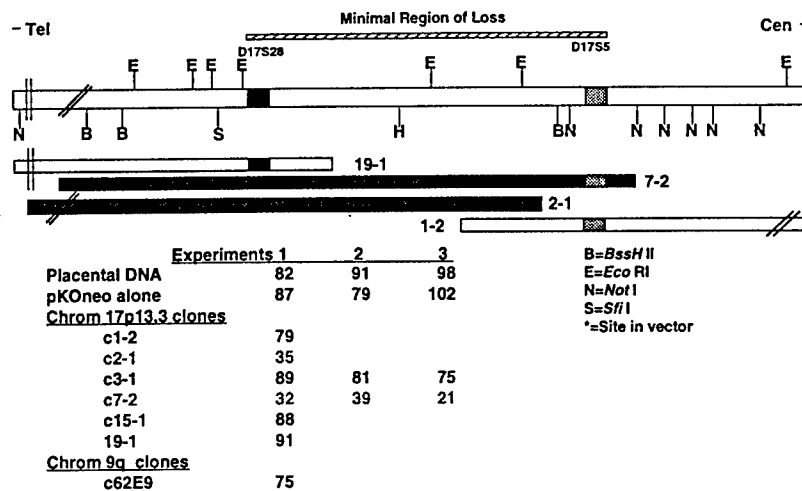
Figure 2. Schematic of chromosome 17p13.3 indicating the minimum region of allelic loss relative to the position of the cosmid clones 7-2 and 2-1, which caused growth suppression when transfected into A2780 cells. Results of the growth suppression experiment are shown in the table at the bottom of the figure. Numbers in the table represent number of colonies formed after three weeks of selection in geneticin, and are the average of three replicates.

Genomic Map of *OVCA1/2*

B=*BssH* II
E=*Eco* RI
N=*Not* I
S=*Sfi* I
*=Site in vector
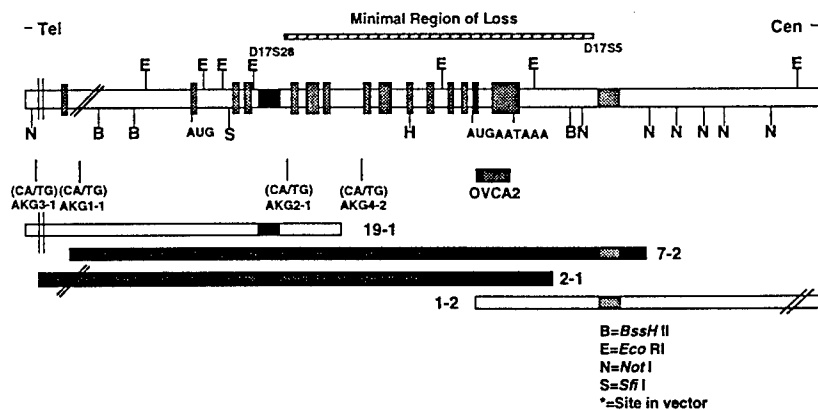
Figure 3. Schematic of chromosome 17p13.3 containing the *OVCA1* and *OVCA2* genes. Stippled boxes correspond to the open reading frames of *OVCA1* and the black box corresponds to the unique exon of *OVCA2*. Cosmid clones, used to identify *OVCA1* and *OVCA2*, which span the minimal region of deletion in ovarian cancer are indicated. Position of D17S5 and D17S28 and three of the four newly identified (CA-GT)$_n$ repeat polymorphisms are indicated relative to *OVCA1*. Lower hatched boxes represent regions of genomic DNA that has been sequenced.
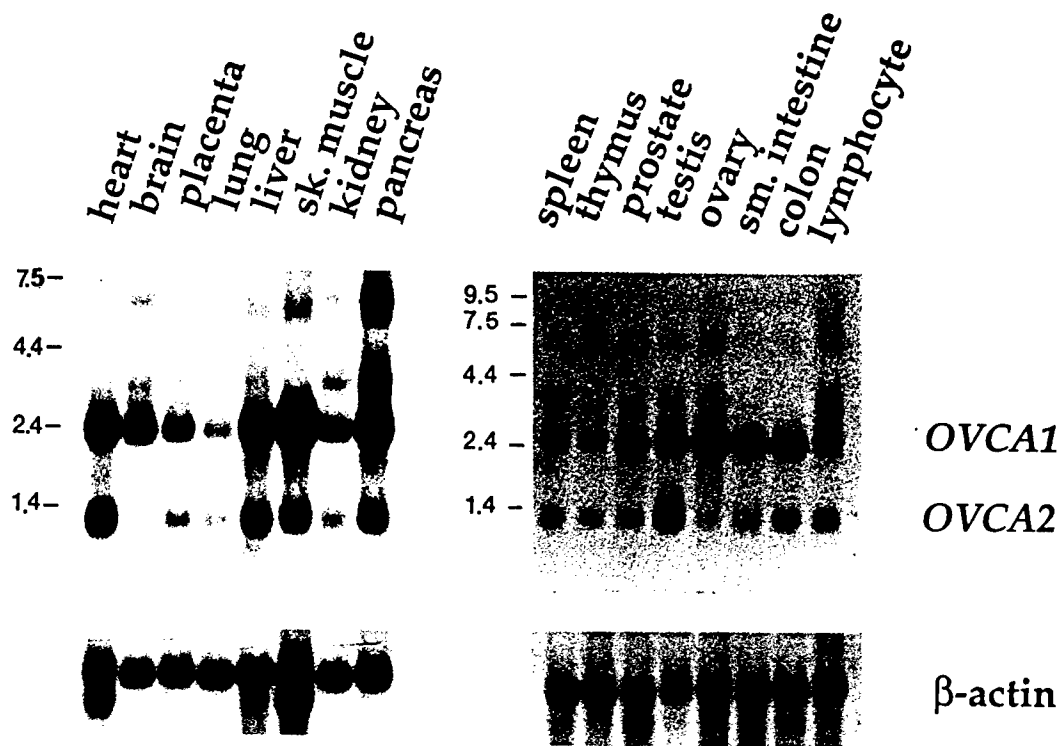
Figure 4. Tissue expression pattern of *OVCA1* and *OVCA2*. Blots containing 5 μg of polyA$^+$ selected mRNA from the indicated human tissues were hybridized with a full-length *OVCA1* cDNA fragment (including OVCA1/OVCA2 shared exon. Size standards are in kilobases. Lower panel; blots were reprobed with β-actin. The heart and skeletal muscle express two forms of β-actin, a 1.8 kb and a 2.0 kb transcript.

Table 1. LOH[1] at Informative Loci on 17p13 in Primary Breast Tumors

	No. of tumors	LOH at OVCA1/2[2]	LOH at TP53[3]
	8	+	–
	1	+	n.d.[4]
	8	+	+
	3	–	+
	16	–	–
Totals	36	17	11

[1]LOH was evaluated by PCR-SSCP analysis using genomic DNA from primary breast tumors.
[2]LOH at at least one of the three (CA-GT)n repeat polymorphisms shown in figure 2.
[3]LOH at a single TP53 intronic repeat polymorphism
[4]not determined

11
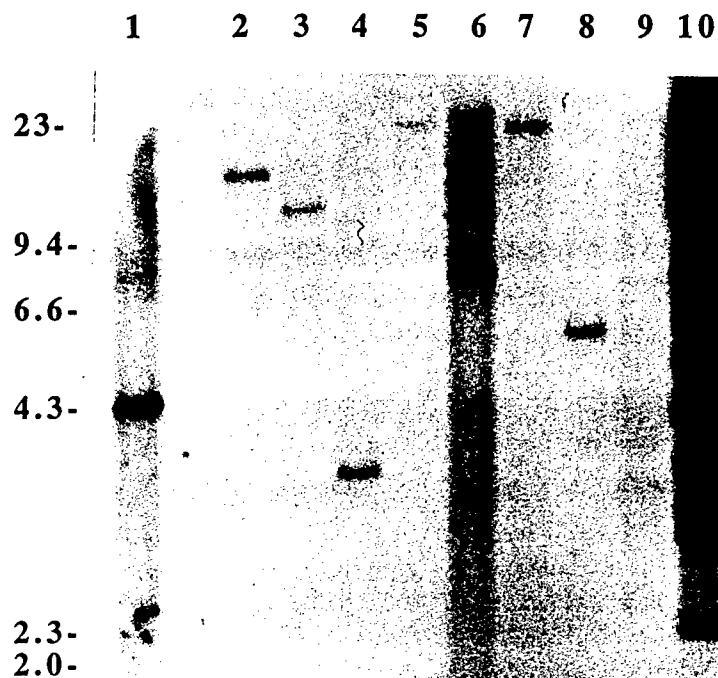
Figure 5. Evolutionary conservation of OVCA2. Southern blot analysis showing cross-species hybridization of an *OVCA2* exon 1 probe (not shared with *OVCA1*) to genomic DNA fragments from various species. Size standards are in kilobases. Legend: lane 1: human, lane 2: bovine, lane 3: cat, lane 4: dog, lane 5: equine, lane 6: monkey, lane 7: mouse, lane 8: porcine, lane 9: rat, lane 10: yeast.

```
                                              ▼PKC▼
      mouse  . . . . . . . . . .  . . . . . . . . M A  A R Q T L R V L C L  A G F R Q S E R G F  R E K T G A L R K A  L R G R A  37
        rat  . . . . . . . . . .  . . . . . . . . . .  . . . V L X L  A G F R Q S E R G F  X E K T G A L R K A  L R G H A  29
       ovca2  . . . . . . . . . .  . . . . . . . . M A  A Q R P L R V L C L  A G F R Q S E R G F  R E K T G A L R K A  L R G R A  37
    c.elegans  . . . . . . . . . .  . . . . . . . . M S  S Q P K L R I L C L  H G Y R Q C D Q S F  R Q K T G S T R K L  V K S L A  37
s.cerevisiae1  . . . . . . . . . .  . . . . . . . . . .  M S E K K K V L M L  H G F V Q S D K I F  S A K T G G L R K N  L K K L G  35
s.cerevisiae2  . . . . . . . . . .  . . . . . . . . . .  . . M T K N V L M L  H G L A Q S G D Y F  A S K T K G F R A E  M E K L G  33
      s.pombe  . . . . . . . . . .  . . . . . . . . . .  M S K P L K V L C L  H G W I Q S G P V F  S K K M G S V Q K Y  L S K Y .  34
         rice  . . . . . . . D G G  X R L G T R L R M E  L D K K F K V L C L  H G F R T S G S F L  K K Q I S K W N P S  I F Q Q .  47
s.cerevisiae3  . . . . . . . . . .  . . . . . . . . . M  T V Q I P K L L F L  H G F L Q N G K V F  S E K S S G I R K L  L K K A N  36
   arabidopsis  G E V P L S L D P L  N Q P S E S N G D N  T R R K L R I L C L  H G F R Q N A S S F  K G R T G S L A K K  L K N I A  55
     Consensus  - - - - - - - - - -  - - - - - - - - - -  - - - - L - V L C L  H G F R Q S - - - F  - - K T G - L R K -  L - - - A  55

                                                      ▼ CK2 ▼         ▼ CK2 ▼
      mouse  E L V C L S G P H P  V P E A A A P E G S  C P D S G P C S P E  E Q P R G C W F S E  E E A D V F S A L E  E P T V C  92
        rat  E L V C L S G P H P  V T E A A A S E G A  G T D S G P C S P E  E Q P R G W W F F E  E E A D V F S A L E  X P T V C  84
       ovca2  E L V C L S G P H P  V P D P P G P E G A  R S D F G S C P P E  E Q P R G W W F S E  Q E A D V F S A L E  E P A V C  92
    c.elegans  E F E F V N G V H S  V A V D E H V D S S  . . . . . . . . . .  . . . R A W W F S N  N E A M S F S S R E  S T E V A  79
s.cerevisiae1  Y D L Y Y P C A P H  S I D K K A L F Q S  E S E K G R D A A K  E F N T S A T S D E  V Y G W F R N P E  S F N S F  90
s.cerevisiae2  Y K L Y Y P T A P N  E F P P A D V P D F  L G E V I A D A P G  D . . . . G E N T G  V L A W . L E N D P  S T G G Y  83
      s.pombe  A E L H F P T G P V  V A D E E A D P N D  E E E K K R L A A L  G . . . G E Q N G G  K F G W . F E V E D  F K N T Y  85
         rice  F D M V F P D G I F  P A G G K S E . . .  . . . . . . . . . I  E . . . G I F P P P  Y F E W . F Q F N K  D F T E Y  86
s.cerevisiae3  V Q C D Y I D A P V  L L E K K D L P F E  M D D E K W Q A T L  D . . . . . . . . A  D V N R A W F Y H S  E I S H E  83
   arabidopsis  E L V F I D A P H .  . . . . . . . . . .  . . E L Q F I Y Q T  A T P P S G A C N K  K F A W L V S S D F  D K P S E  97
     Consensus  E - - - - - - - H -  V - - - - - - - - -  - - - - - - - - - -  E - - - G - - - - -  - - A W - F S - - E  - - - - -  110

              ▼        pseudo — leu              ▼                    ▲ myb — DNA      ▲
      mouse  S G L Q E A L E T V  A R A L D T L W P F  D G L L G . . . . .  . . . . . . . . . .  . . . . . . . . . .  . x x x P  121
        rat  R G L E A A L E T V  A Q A L D K L G A F  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . .  104
       ovca2  R G L E E S L G M V  A Q A L N R L G P F  D G L L G F S Q G A  A L A A L V C A . .  . . . L G Q A G D P  R F P L P  142
    c.elegans  V G F E E S V A A V  V K F I E E N G P F  D G L L G F S Q G A  S M V H L L I A . .  . . . K A Q L G E I  K L P A L  129
s.cerevisiae1  Q I D Q K V E N Y L  R N Y V L E N G P F  D G V I G F S Q G A  G L G G Y L V T D F  N R I L N L T D . E  Q Q P A L  144
s.cerevisiae2  F I P Q T T I D Y L  H N Y V L E N G P F  A G I V G F S Q G A  G V A G Y L A T D F  N G L L G L T T . E  E Q P P L  137
      s.pombe  G S W D E S L E C I  N Q Y M Q E K G P F  D G L I G F S Q G A  G I G A M L A Q M L  H A G Q P P N P Y V  Q H P P F  140
         rice  T N L D E C I S Y L  C D Y M V K N G P F  D G L L G F S Q G A  T L S A L L I G Y Q  A Q G K V L N . . .  D H P P I  138
s.cerevisiae3  L D I S E G L K S V  V D H I K A N G P Y  D G I V G F S Q G A  A L S S I I T N K I  S E L V P . . . . .  D H P Q F  133
   arabidopsis  T G W D S G P M P V  R S I G S N Q T Q T  X R V L I K S V . .  . . . . . . . . . .  . . . . . . . . . .  . . . . .  125
     Consensus  - G - - E - L - - V  - - - - - - N G P F  D G L L G F S Q G A  - - - - - L - - - -  - - - - - - - - - -  - - P - -  165

                                                                          ▼PKC ▼
      mouse  R F I I L V S G S C  P R . . G L K E P I  L Q T P . . . . . .  . . . . . . . M S L  P S L H V F G D T D  R V I P S  161
        rat  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . .  104
       ovca2  R F I L L V S S F C  P R G I G F K E S I  L Q R P . . . . . .  . . . . . . . L S L  P S L H V F G D T D  K V I P S  184
    c.elegans  R F A I F F S G F L  S L S S K H D S L T  L L R I . . . . . .  . . . . . . . K E F  P S M H V F G D A D  E I V A R  171
s.cerevisiae1  K F F I S F S G F K  L E D Q S Y Q K E Y  H . R I . . . . . .  . . . . . . . I Q V  P S L H V R G E L D  E V V A E  185
s.cerevisiae2  E F F M A V S G F R  F Q P Q Q Y Q E Q Y  D L H P . . . . . .  . . . . . . . I S V  P S L H V Q G E L D  T I T E P  179
      s.pombe  K F V V F V G G F R  A E K P E F D H F Y  N P K . . . . . . .  . . . . . . . L T T  P S L H I A G T S D  T L V P L  181
         rice  K . . S W L N S G R  S S G P K I . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . .  152
s.cerevisiae3  K V S V V I S G Y S  F T E P D P E H P G  E L R I T E K F R D  S F A V K P D M K T  K M I F I Y G A S D  Q A V P S  188
   arabidopsis  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . .  125
     Consensus  - F - - - - S G F -  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  P S L H V - G - - D  - - - - -  220

      mouse  Q E S M Q L A S R F  . . . . . . . . L G  A V T L T H S G G H  F I P A A A S Q R Q  A Y L K F L D Q F A  E . . . . 204
        rat  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . 104
       ovca2  Q E S V Q L A S Q F  . . . . . . . . P G  A I T L T H S G G H  F I P A A A P Q R Q  A Y L K F L D Q F A  E . . . . 227
    c.elegans  P K S E K M A D M F  . . . . . . D V E  P L R I A H D G G H  V V P S M S K H K E  K I A G F M R E Q L  D R K I E 219
s.cerevisiae1  S R I M A L Y E S W  . . . . . . P D N K  R T L L V H P G A H  F V P N S K P F V S  Q V C N W I Q G I T  S K E G Q 234
s.cerevisiae2  A K V Q G L Y N S C  . . . . . . T E D S  R T L L M H S G G H  F V P N S R G F V R  K V A Q W L Q Q L T  . . . . . 223
      s.pombe  A R S K Q L V E R C  . . . . . . E N A H  . . V L L H P G Q H  I V P Q Q A V Y K T  G I R D F M F S A P  T K E P T 228
         rice  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . 152
s.cerevisiae3  V R S K Y L Y D I Y  L K A Q N G N K E K  V L A Y E H P G G H  M V P N K K D I I R  P I V E Q I T S S L  Q E A S E 243
   arabidopsis  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . 125
     Consensus  - - S - - L - - - -  - - - - - - - - - -  - - - L - H - G G H  - V P - - - - - - -  - - - - - - - - - -  - - - - - 275
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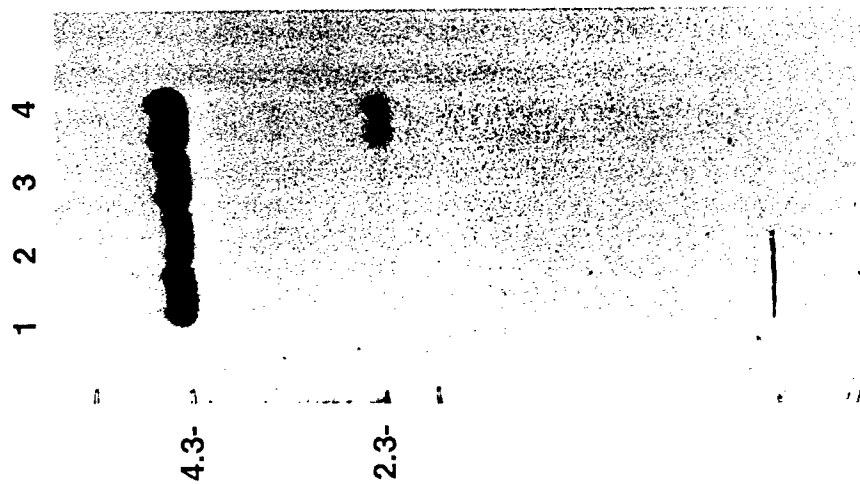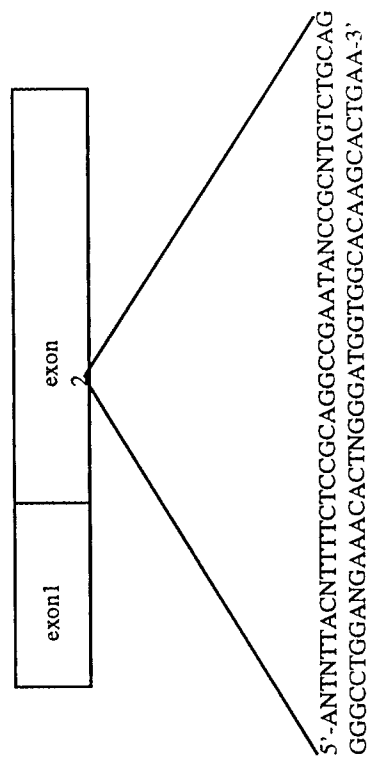
Figure 6. Multiple sequence alignment between OVCA2 amino acid sequence, and similar sequences from mouse, rat, yeast, nematode, rice and arabidopsis. Two protein kinase C phosphorylation sites (PKC), two casein kinase-2 phosphorylation sites (CK2), the potential pseudo-leucine zipper motif (p-leu), and the potential myb DNA binding site (myb-DNA) are all indicated.

13

Figure 8. Southern blot of EcoRI digested genomic DNA from blood lymphocytes (lanes 1-3) or invasive ductal carcinoma of the breast (lane 4) from the same patient. The presence of a second band in the tumor DNA confirms the insertional mutation observed in exon 2 of OVCA2.
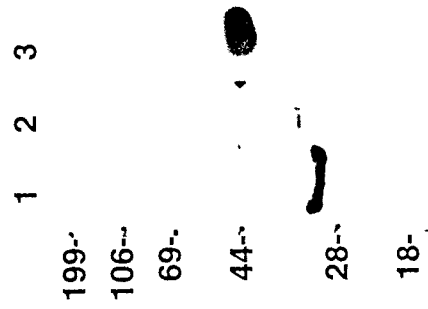
OVCA2 Insertional Mutation

5'-ANTNTTACNTTTTCTCCGCAGGCCGAATANCCGCNTGTCTGCAG
GGGCCTGGANGAAACACTNGGGATGGTGCCACAAGCACTGAA-3'

Figure 7. Insertional mutation in exon 2 of OVCA2 in invasive ductal carcinoma of the breast. The mutation was identified using PCR-SSCP analysis and sequencing.

Figure 10. Immunofluorescent staining of HA-OVCA2 transfected Cos cells using HA Abs. Cells were grown on coverslips, transiently transfected with HA-OVCA2, then stained two days later. Staining was performed with (a) 1:1000 HA mAb, followed by 1:100 FITC-conjugated antimouse secondary Ab, (b) 1:1000 HA pAB, followed by 1:1000 biotinylated antirabbit secondary Ab, and 1:1000 streptavidin-Texas Red conjugate. Note both cytosolic and nuclear localization of OVCA2. 400x magnification.

Figure 9. Western blot analysis using OVCA2 Ab. 25µg of protein were loaded from HA-OVCA2 transfected Cos cells (lane 1), untransfected Cos cells (lane 2) or bacteria expressing GST-OVCA2 (lane 3). Ab was immunoaffinity purified, and used at a 1:10 (lanes 1,2) or 1:100 (lane 3) dilution. Markers are in kilodaltons.

Figure 11. Mutational analysis of the *BRCA1* gene using a Mismatch Detection Analysis (MDA) assay. Representative electrophoretograms corresponding to the analysis of *BRCA1* exon 11 PCR products for individual 2651-1 (upper panels), and for three unrelated individuals (lower panels). **Upper panels**; DNAs were amplified using fluorescently labeled exon 11D primer set. Heteroduplexes were formed between fluorescent-labeled PCR products amplified from constitutional DNA and the duplexes were treated with CEL I and analyzed for incised bands. The absorbance of the FAM-labeled strands (blue) was measured using an excitation wavelength of 488nm and bandpass filter for detection at 531nm. Blue peaks correspond to full-length product (~1.1kb) unused primers (20 to 100 bases), and a cut produced by the enzyme (at nucleotide 643). The absorbance of the TET-labeled strands (green) was measured using an excitation wavelength of 488nm and bandpass filter for detection at 545nm. Green peaks correspond to full-length product (1.1kb) unused primers (20 to 100 bases), and a cut produced by the enzyme (at nucleotide 484). Sequence analysis of this individual indicated a five base pair deletion at nucleotide 3819 (3819del5) of the *BRCA1* cDNA. The internal standard DNA molecular weight markers labeled with TAMRA was used to determine the band sizes generated for each MDA reaction. **Lower panels**; FAM-labeled exon 11A PCR for individuals HO-502, 71-8142, and 436-2975. Peaks correspond to full-length product (1043 bases), unused primers (20 to 50 base), and cuts produced by the enzyme at base 183 (panels A and B) and at base 486 (panel B only). Sequence analysis revealed sequence alterations at 2430 (C--T) and at 2731 (C--T). Both alterations (Leu771Leu and Pro871Leu) were determined to be common sequence polymorphisms. These results demonstrate that MMR endonuclease analysis can readily detect multiple sequence alterations (single base substitutions and DNA loops) in large fragments of DNA.

Lane 10: 6-FAM 1100 bp Exon 11 2651-1 mutation 3819 del 5

Lane 10: TET 1100 bp Exon 11 2651-1 mutation 3819 del 5

Lane 10: tamra internal lane size standard

Lane 2: 6-FAM Exon 11A 1043 bp HPB-502 with 2 polymorphisms

Lane 3: 6-FAM Exon 11A 1043 bp 71-8142 with 1 polymorphism

Lane 7: 6-FAM Exon 11A 1043 bp 436-2975 without polymorphism

Table 2: Synopsis of Yeast Two Hybrid Results with OVCA2

β-Galactosid-ase (+)[1]	Leucine (-) [2]	Double Positives	Unique Strong Interactors[3]
124	136	83	54

1. Colony turned blue in the presence of Xgal due to induction of β-Galactosidase expression
2. Colony grew in the absence of leucine due to induction of Leu2 expression
3. Colony turned blue and grew in the absence of leucine;co-induction of LacZ and Leu2 expression

Table 3: Candidate Interactors with OVCA2

1   Beta Tubulin
2   Alpha Tubulin
3   Human Rb Binding Protein (RbAb46)
4   Proliferation Associated Gene
5   Cytosolic Acetoacetyl Coenzyme A Thiolase
6   Human VH-L Binding Protein 1 mRNA
7   Hemapoietic Proteoglycan Core Protein
8   Human Secretory Granule Proteoglycan Core Protein
9   DNA J Protein
10 Human Complement Component C3 mRNA
11 H.Sapiens Splicing Factor mRNA
12 Human mRNA for Heat Shock Protein hsp86
13 H.Sapiens Mitochondrial hsp75 mRNA
14 H.Sapiens Cosmid HTGS Phase 3
15 H.Sapiens Y Chromosome Cosmid AMF containing Yp Pseudo
    Autonomal Boundary
16 H.Sapiens mRNA for Calcium Binding Protein S100P
17 Human Calcium Modulating Cyclophilin Ligand mRNA
18 Human Cyclophilin 40 mRNA
19 Human Eukaryotic Translation Initiation Factor
20 Human Aldehyde Dehydrogenase
21 Human mRNA Precursor of EGF Receptor
22 Human mRNA for Protein D123

CONCLUSIONS:

In order for future therapies to be developed for the fight against cancer it is important to understand the basic molecular mechanisms that give rise to a specific cancer type. The fundamental mechanisms underlying the genetic basis of cancer are slowly being defined and involve alterations in genes which have been classified into three general categories: (i) protooncogenes are involved in growth promotion and the defects leading to cancer are a gain of function; (ii) tumor suppressor genes are negative regulators of growth and a loss of function gives rise to cancer; and (iii) DNA repair genes are involved in maintaining the fidelity of the genome and altered function can lead to increase rates of mutations in both classes of cancer-causing genes. Cancer is a multistep process that involves alterations in many specific genes. The normal cell has multiple independent mechanisms that regulate its growth and differentiation and several separate events are required to override these control mechanisms. Progress is now being made in isolating these genes and the proteins they encode for, determining the normal cellular functions of the proteins and in investigating the mechanisms of tumorigenesis.

Breast cancer is a very common disease, causing about 10% of deaths in women in the Western World (22). Molecular genetic analysis of breast tumors has revealed many genetic aberrations that may represent important steps in tumor development. To understand the genetic pathways underlying breast tumor development, it is necessary to identify the genes affected by these genetic aberrations and establish any correlations between disruption of their function and tumor phenotype.

Chromosome 17 frequently shows loss of heterozygosity (LOH) in breast carcinomas (2, 3, 5, 13, 15, 23). In addition, re-introduction of chromosome 17 fragments into breast cancer cell lines has been shown to suppress tumorigenicity (24). Therefore, inactivation of tumor suppressor genes on chromosome 17 appears to be a critical event in the pathogenesis of breast cancer. Although *TP53* at chromosome 17p13.1 is involved in the pathogenesis of breast cancer, LOH mapping studies in breast, ovarian and brain carcinomas have defined a region distal to *TP53*, at 17p13.3, thought to harbor a tumor suppressor gene (2, 3, 5, 13, 15, 23). In addition, a fragment containing 17p13.3 has been shown to suppress the tumorigenicity of breast cancer cell lines (25).

OVCA2 is a novel gene that has recently been isolated and maps to chromosome 17p13.3 (17). OVCA2 is highly conserved and this suggests that it has an important cellular function. Reduced levels of expression of OVCA2 have been observed in breast and ovarian tumors compared to primary human mammary epithelial and ovarian surface epithelial cells. Through limited screening of breast tumors for mutations in OVCA2, one sequence alteration has been detected that would resulting in an impaired OVCA2. Furthermore, OVCA2 binds to several proteins with important cellular functions, suggesting a central role in control of cell growth and morphology. This combined evidence suggests that OVCA2 has an important function, the disruption of which may lead to tumorigenesis. The tumor suppressor properties and normal cellular function of OVCA2 therefore warrant further investigation.

We have addressed portions of all three **Specific Aims** during the current year and have initiated several new projects all directed towards furthering our understanding of the role of OVCA2 in the development of cancer. In this aspect, we will continue to 1) evaluate accrued normal/tumor pairs for LOH at 17p13.3; 2) search for sequence alterations at the genomic level by MDA; 3) evaluate the protein extracts from tumors demonstrating LOH at 17p13.3 for expression of OVCA2 by western blotting using newly derived antibodies; 4) determine if exogenous expression of OVCA2 leads to growth suppression in a panel of tumor cell lines; 5) examine *OVCA2* transfectants for the loss of features associated with transformation; 6) continue to raise antibodies to OVCA2 that can routinely be used for immunohistochemistry and immunoprecipitations; and 7) investigate the biochemical mechanism of action of the OVCA2 protein by studying OVCA2 protein interactors and the effects of disrupting these normal interactions *in vivo*.

Overall, we feel that the recent discoveries presented above and the studies proposed will continue to enhance our understanding of the molecular genetic events involved in the development of breast cancer, as well as potentially other neoplasias and genetic disorders involving chromosome 17p13.3.

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